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SOD1 plasma level as a biomarker for therapeutic failure in cutaneous leishmaniasis

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ABSTRACT

Here we show that increased plasma SOD1 level significantly predicts therapeutic failure to pentavalent antimony in cutaneous leishmaniasis caused by *Leishmania braziliensis.* In *Leishmania amazonensis*-infected patients, host SOD1 levels discriminate between localized and drug-resistant diffuse cutaneous leishmaniasis. Using *in situ* transcriptomics (nCounter), we demonstrate a significant positive correlation between host SOD1 and IFN-alpha/beta mRNAs, as well as interkingdom correlation between host SOD1 and parasite SOD2/4 mRNAs. In human macrophages, SOD1 *in vitro* treatment increases parasite burden and induces diffuse cutaneous leishmaniasis-like morphology. Thus, SOD1 is a clinically relevant biomarker and a therapeutic target in both localized and diffuse cutaneous leishmaniasis.

INTRODUCTION

Leishmaniasis is endemic in several parts of the world, with a global prevalence of over 12 million cases. Mainly classified in two distinct clinical forms, leishmaniasis can affect the skin (cutaneous leishmaniasis-CL) or viscera (visceral leishmaniasis-VL). Cutaneous leishmaniasis, with 1.5 to 2 million new cases per year, is an emerging infectious disease in several countries, due to behavioral and environmental changes, as well as HIV co-infection (1). The clinical spectrum of CL, ranges from the mild cutaneous form (localized cutaneous leishmaniasis; LCL) to the disfiguring diffuse cutaneous leishmaniasis (DCL) and mucosal form (mucocutaneous leishmaniasis; MCL). *Leishmania (L.) braziliensis* causes LCL and MCL, whereas *L. amazonensis* causes LCL and, sporadically, DCL (2-4). A low parasite load and a variable tendency for self-healing characterize LCL, but treatment is strongly recommended to reduce scarring and prevent progression to severe MCL. Pentavalent antimonials (Sb^V) are currently the first-line treatment for LCL, nonetheless, 24.4% of the treated patients fail to cure with a single cycle of Sb^V and a second or more cycles are indicated to heal the lesions (5). On the other hand, multiple non-ulcerative nodules with extremely high parasite burden, as well as a non-healing or relapsing phenotype, characterize DCL patients (1, 3, 4, 5). DCL is typically refractory to Sb^V and second-line treatment. Recently, miltefosine treatment induced clinical improvement and reduced parasite burden in a cohort of DCL patients, nevertheless treatment suspension lead to relapse (6).

As an obligatory intramacrophage parasite, *Leishmania spp.* developed several evasion mechanisms to avoid human macrophage leishmanicidal activity. One of such mechanisms

may involve superoxide dismutase (SOD), a conserved isoenzyme responsible for detoxifying reactive superoxide both in *Leishmania* and human organisms. Previous studies have demonstrated that SOD-deficient New World *Leishmania* species as well as Old World Leishmania have enhanced sensitivity to exogenous superoxide in axenic culture and reduced survival in mouse and human macrophages (7, 8). We have previously shown an IFN-beta-induced SOD1-mediated inhibition of superoxide-dependent parasite killing in *Leishmania*-infected human macrophages in vitro (9). In addition, CL patients have increased SOD activity in plasma as compared to normal subjects (10). We hypothesized that plasma levels of host SOD1 might reflect clinical status in CL, and might therefore represent a candidate biomarker.

Patients and Methods

This study was approved by the Ethics Committee of the Gonçalo Moniz Research Center. Informed consent was obtained from all patients and healthy controls. Cutaneous leishmaniasis patients were diagnosed as described (11), according to characteristic lesion morphology, positive skin test, seropositivity towards *Leishmania* antigen and/or the presence of parasites in the lesion. LCL patients infected with *Leishmania braziliensis* (n=58, 27 male, 29.6±2.3 years) were recruited and treated in two outpatient clinics (Jequié and Jiquiriçá-BA, NE Brazil) covering the same rural area. LCL patients infected with *L amazonensis* (n=10, 6 male, 38.8±6 years) were recruited and treated at the Professor Edgar Santos University Hospital (Salvador-BA, NE Brazil). Plasma samples from LCL patients were collected at diagnosis (before treatment). In addition, a paired sample was obtained from 13 patients at the time of definite clinical cure (range 68-417 days). LCL patients (both *L. braziliensis*- and *L. amazonensis*-infected) received standard intravenous

pentavalent antimony (Glucantime; Rhodia, 20 mg/kg/day for 20 days). Cure was defined by the complete scarring of lesions, without induration and without relapse during two years of follow-up. Although all *L. amazonensis* patients cured within 90 days following a single treatment cycle, *L.braziliensis* patients needed 190.5 ± 16.0 days and 2.2 ± 0.2 treatment cycles to cure, with 23/58 patients (39.7 %) classified as failing (>2 cycles). DCL patients (n=8, 3 male, 31.5 ±7.1 years) infected with *L. amazonensis* were recruited and treated at the Presidente Dutra University Hospital- HUPD (São Luís-MA, NE Brazil). DCL patients had mean disease duration of 10.9 ±2.1 years, during which several therapeutic schemes (mean of 2.7±0.9 different schemes/patient, including liposomal amphotericin B and combinations of Glucantime, IFN-γ and Pentamidine) were applied, either without or with only a transient clinical effect. Since DCL is a rare clinical manifestation, no samples from untreated patients (at diagnosis) were available for comparison. SOD1 plasma levels were quantified using a human SOD1 ELISA Kit (Calbiochem). RNA was extracted from skin biopsies of LCL and DCL lesions, as well as healthy controls, using Trizol, followed by an additional purification using RNeasy (QIAgen Benelux B.V., Venlo, the Netherlands). Using in situ transcriptomics, we simultaneously quantified host and parasite RNAs in skin biopsies from LCL and DCL patients, as well as healthy controls, by nCounter technology (NanoString, Seattle), based on molecular bar-coding of target RNA transcripts and digital detection at the femtomolar range using direct hybridization, without reverse transcription nor amplification (12). Human SOD1, SOD2, SOD3, IFNA1, IFNA2, IFNA4, IFNB1 and *Leishmania braziliensis* and *amazonensis* SOD1-5 mRNAs were quantified *in situ*, in addition to several housekeeping genes for normalization (GUSB, G6PD, GAPDH, HPRT1), as well as

leukocyte-specific genes (CD3, CD14, CD19, CD56, CD45). Quantification of parasite burden in vitro was carried out as described (9). Briefly, monocyte-derived human macrophages were infected with *L. amazonensis* (MHOM/BR/87/BA125) and treated with SOD1 (Sigma) for 48h, followed by extensive washing, staining with hematoxylin/eosin and counting of intracellular amastigotes (100 cells, duplicates for each sample). Parametric and non-parametric tests were used according to Kolmogorov-Smirnov test for normality. For multiple comparisons, Kruskal-Wallis with Dunn's post-test One-way ANOVA with post-test for linear trend were used. For comparison between two groups, F-test, Mann Whitney, t test or Wilcoxon tests were used. To identify biomarkers, Receiver Operating Characteristic (ROC) curve was used. All tests were two-tailed and differences were considered significant at P values <0.05.

Results

We measured plasma levels of SOD1 in healthy controls, LCL patients (*L. braziliensis* or *L. amazonensis*-infected patients) and DCL patients (all infected with *L. amazonensis*) and investigated its potential role as a biomarker. SOD1 level increased three-fold from 30.2± 3.9 pg/ml in healthy controls to 90.6±8.0 pg/ml in LCL patients infected with *L. braziliensis*, (Kruskal-Wallis p<0.01, Dunn's post-test p<0.001<0.0001), and 3.1 fold to 93.1±24.7 pg/ml in LCL patients infected with *L. amazonensis* (Kruskal-Wallis p<0.01, Dunn's post-test p<0.01). On the other hand, SOD1 levels were further increased 4.4 fold from 93.1±24.7 pg/ml in LCL patients infected with *L. amazonensis*) to 407.3±88.0 pg/ml in DCL patients, (Mann-Whitney test, p=0.0005) (Fig. 1A). To investigate whether increased SOD1 in LCL is related to active disease, SOD1 was also measured in paired samples of LCL (*L. braziliensis*) patients (n=13), at pre-treatment (diagnosis) and post-

treatment (definite clinical cure). SOD1 levels decreased 1.6 fold from 99.2±20.81 pg/ml to 60.4±5.2 pg/ml in in pre- vs. post-treatment samples (Wilcoxon matched pairs test, p=0.027, Supplementary Figure 1A). Since variances differed significantly between controls and LCL patients infected with *L. braziliensis*, (F-test, p<0.0001), we hypothesized that heterogeneous SOD1 levels in LCL patients might reflect differences in clinical status and/or therapeutic response. SOD1 levels did not significantly correlate with age, gender, disease duration, lesion size or healing time of LCL patients (data not shown). However, SOD1 plasma levels were significantly associated with therapeutic response, quantified as number of treatment cycles (Kruskal-Wallis p=0.0069, Dunn's post-test p<0.001, Fig. 1C). Using Receiver Operating Characteristic (ROC) curve analysis, successful (1 cycle) and failing (>2 cycles) therapeutic response could be significantly discriminated through SOD1 plasma levels (ROC AUC 0.83, p=0.00025), authenticating its clinical utility as a biomarker (Fig. 1D). Above a cut-off of 122 pg/ml, 9 out of 22 failing patients (40.9 %) could be identified with 100% specificity, i.e. none of the patients cured with 1 treatment cycle displayed SOD1 levels above 122 pg/ml. Strikingly, at a similar cut-off of 112 pg/ml, DCL patients could be discriminated from treatable LCL patients (*L. amazonensis*) with 100% sensitivity and 90% specificity (ROC AUC 0.95, p=0.001; Fig. 1B).

To test the hypothesis whether these high levels of SOD1 in plasma of CL patients might be directly related to increased parasite burden, human macrophages from healthy donors were infected with *L. amazonensis* (5:1) and cultivated in the absence or presence of recombinant SOD1 protein. As shown in Fig. 2A, SOD1 treatment significantly increased parasite load (t test, p=0.042). Moreover, infected human macrophages treated with recombinant SOD1

protein presented cytomorphological features (Fig. 2B) strikingly similar to the vacuolized and highly parasitized macrophages from cutaneous lesions of DCL patients (Fig. 2C).

Finally, using *in situ* transcriptomics (nCounter), we demonstrate a significant positive correlation between host SOD1 mRNA level and parasite SOD2 (p=0.019, r=0.98) and SOD4 (p=0.0026, r=0.99) mRNA in diffuse leishmaniasis. Taken together, these data suggest that systemic SOD1 might play a causal role in both the clinical and anatomo-pathological phenotype of DCL.

Discussion

Up to now, no non-invasive biomarker for therapeutic response to Sb^V has been described in cutaneous leishmaniasis. In the present work, we demonstrate host SOD1 as a biomarker for therapeutic failure of first-line Sb^V in New World CL patients. SOD1 plasma levels could significantly discriminate between successful and failing therapeutic response in LCL patients (*L. braziliensis*) and between treatable LCL patients (*L. amazonensis*) and refractory DCL patients. Quantification of SOD1 plasma level at diagnosis represents a rapid, sensitive and inexpensive new tool in the clinical management of CL. Further research will be necessary to test its predictive value in CL caused by other *Leishmania* species, since in Peru therapeutic failure to antimonial therapy is significantly associated with *L.braziliensis* and *L. peruviana*, but not with *L. guyanensis* (5). By identifying, at the time of diagnosis, up to 38.5 % of patients (with 100% specificity) who will fail antimonial therapy (Fig.1C), second-line treatment (e.g. amphotericin B, miltefosine) could be provided as a first choice, thereby diminishing public health costs and avoiding

unnecessary and prolonged exposure of the patients to the often severe side effects of intravenous pentavalent antimony.

Although SOD1 is an intracellular antioxidant enzyme, our results suggest a direct role for increased systemic (plasma) SOD1, as a trigger of parasite replication in infected macrophages. Likewise, treatment of infected human macrophages with purified SOD1 in vitro lead to strongly increased parasite burden and the appearance of large parasitophorous vacuoles, reminiscent of typical nodular lesions in diffuse leishmaniasis (13). Moreover, this correlation between SOD1 and parasite burden was recapitulated in situ in DCL lesions, as evidenced by a strong linear relationship (r=098-0.99) between host (human SOD1) and parasite (*L. amazonensis* SOD2 and SOD4) superoxide dismutases. This parallel cross-regulation at the transcriptional level between superoxide-quenching enzymes that are hundreds of million years distant in evolution is a striking example of interkingdom signaling. To our knowledge, this is the first description of interkingdom signaling in protozoan-animal interaction. In keeping with our cytomorphological data (Fig. 2B), this phenomenon is highly specific for cytosolic SOD1, since cross-kingdom correlations were not observed for mitochondrial SOD2 or extracellular SOD3 with any of the parasite mRNAs. The extremely long (mean disease duration of 10.9 ±2.1 years) uncontrolled parasite replication in DCL patients might have lead to selection of SOD-overexpressing parasite clones (7, 8). Alternatively, increased systemic levels of SOD1 in LCL and DCL patients might reflect a parasite escape mechanism from host leishmanicidal activity mediated by type I IFN, as suggested by our previous work (9) and recently confirmed in murine and human macrophages (14). In agreement with this hypothesis, we found a

significant positive correlation between SOD1 mRNA and total IFN-alpha/beta mRNA levels using in situ transcriptomics (nCounter, Supplementary Figure 2A).

Finally, since all our DCL patients also failed multiple other treatment schemes (including pentamidine, amphotericin B and IFN-gamma, alone or in combination), increased plasma SOD1 truly reflects broad therapeutic failure. The dramatic increase in DCL also reinforces the idea that SOD1 is not a mere 'surrogate' marker, but a key molecule in DCL pathogenesis, driving perennial parasite replication and systemic dissemination, as suggested by Fig. 2A-D. Hence, SOD1 is also a candidate therapeutic target in CL, reinforcing our previous suggestion of pharmacologic SOD1 inhibition by DETC (15) as a viable therapeutic alternative in LCL and DCL in particular, for which no effective treatment options exist.

In conclusion, our results indicate host SOD1 as a clinically useful biomarker for therapeutic failure, as well as a possible therapeutic target, in localized and diffuse cutaneous leishmaniasis.

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Figure Legends

Figure 1: SOD1 is a biomarker for therapeutic failure in cutaneous leishmaniasis. (A) SOD1 measurements in plasma samples of healthy controls (n=20), LCL patients (*L. braziliensis*) (n=58), LCL patients (*L. amazonensis*) (n=10) and DCL patients (*L. amazonensis*) (n=8). Box-whiskers plots represent the median and interquartile range of each group (Kruskal-Wallis test $p<0.001$, Dunn's post-test, healthy controls vs. LCL patients (*L. braziliensis*) ***$p<0.001$, healthy controls vs. LCL patients (*L. amazonensis*) **$p<0.01$, and Mann Whitney test, LCL patients (*L. amazonensis*) vs. DCL ***$p<0.001$). (B) Plasma SOD1 level significantly discriminates LCL from DCL patients (all infected by *L. amazonensis,* ROC AUC 0.95, ***$p=0.001$). (C) SOD1 measurements in plasma samples of LCL patients (*L. braziliensis*) classified according to number of treatment cycles (Kruskal-Wallis $p=0.0069$, Dunn's post-test **$p<0.001$), three outliers (Tukey's test) are shown as single dots. (D) Plasma SOD1 level significantly predicts therapeutic response in LCL patients (*L. braziliensis*) (Success =1 treatment cycle, Failure >2 treatment cycles) (ROC AUC 0.83, ***$p=0.00025$, without outliers as in (C)).

Figure 2: SOD1 correlates to parasite burden in vitro and in situ in DCL. (A) and (B) Human macrophages were infected with *Leishmania amazonensis* (5:1 ratio) for 4h and then treated in the absence or presence of SOD1 (175 U/ml) protein for 48h. Cells were fixed on glass slides and stained with hematoxylin and eosin. (A) Parasite burden was quantified, each bar represents the mean ± SEM of 3 donors (t test, *$p=0.042$). (B) Infected macrophages (1000x magnification) left untreated (left panel) or SOD1-treated (right panel). (C) Cutaneous lesion biopsy of a representative DCL patient stained with hematoxylin and eosin (1000x magnification). (D) Positive correlation between human

SOD1 and parasite SOD2 (Pearson correlation, r=0.98, p=0.019) and SOD4 (Pearson correlation, r=0.99, p=0.0026) mRNA levels (normalized to G6PD), quantified by nCounter in situ in DCL biopsies (n=4).